

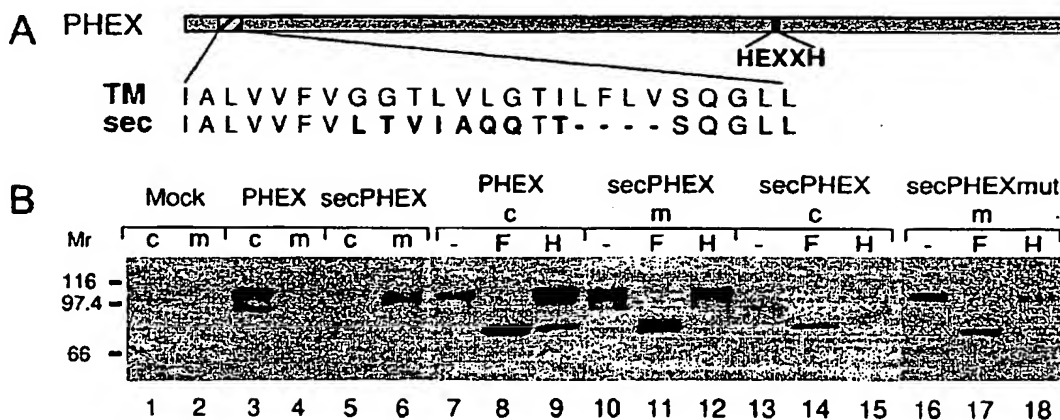
(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
28 February 2002 (28.02.2002)

PCT

(10) International Publication Number
WO 02/15918 A2

- (51) International Patent Classification⁷: A61K 38/00
- (21) International Application Number: PCT/CA01/01220
- (22) International Filing Date: 23 August 2001 (23.08.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/227,012 23 August 2000 (23.08.2000) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD AND COMPOSITIONS FOR PROMOTING OSTEOGENESIS



(57) Abstract: The object of the present invention is to increase PHEX activity to improve the osteogenic process in a mammal where this process has been diminished due to pathology or a condition that require osteogenesis. Osteocalcin is an endogenous inhibitor of the PHEX activity. Therefore, the identification of a substance capable of potentiating PHEX activity by preventing the inhibitory action of endogenous inhibitors will improve osteogenesis. Since PHEX is generally associated with the growth plane of bone or teeth and the absence of osteocalcin is associated with increased bone mass, potentiation of PHEX activity can promote bone growth. Increased PHEX activity can also be obtained by administration of the enzyme itself.

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TITLE OF THE INVENTION

Method and Compositions for Promoting Osteogenesis

5 FIELD OF THE INVENTION

The present invention relates to a novel process for promoting the osteogenic process, the deficiency of which is attributable to various pathologies. More specifically, the present invention is focused on the
10 enhancement of osteogenesis by increasing the activity of PHEX, either by administering PHEX or by increasing the activity of existing PHEX by inhibiting osteocalcin. The present invention further provides a method for screening agents capable of abolishing the inhibitory action of osteocalcin.

15 BACKGROUND OF THE INVENTION

The bone. The solid matrix of the bone is made of an organic phase, collagen, and of an inorganic phase, composed of calcium and phosphate (hydroxyapatite). Bones are continuously remodeling themselves, a
20 process of combined dissolution (resorption) and re-construction of the bone matrix. The bone relies on two cell types for remodeling process: osteoclasts, which are responsible for resorption, and osteoblasts, which promote for bone formation. In normal bones, both processes are strictly coordinated to maintain bone mass within defined limits. More specifically,
25 at a specific site, resorption occurs first and over a shorter period than bone formation. At the end of the formation stage most osteoblasts will disappear through the apoptotic process. About 10% will be enclosed in the bone and remain there to form osteocytes. It is generally believed that the osteocytes participate in some form of mechanical stress sensing
30 mechanism. Osteoporosis occurs when the resorption process is initiated more often than formation is completed, leading progressively to more fragile bones (Harrison 14th Ed).

PHEX. The PHEX gene (formerly PEX; a Phosphate regulating gene with homologies to Endopeptidases on the X chromosome) was identified by a positional cloning approach as the candidate gene for human X-linked hypophosphatemia (XLH) (1). XLH is a Mendelian disorder of phosphate homeostasis characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, and renal defects in phosphate re-absorption and vitamin D metabolism (2). Several groups have cloned and sequenced the human and mouse PHEX/Phex cDNAs (3-7) (PHEX/Phex refers to the human and mouse genes, respectively). Amino acid sequence comparisons have demonstrated homologies between PHEX/Phex protein and members of the M13 endopeptidase family, as previously observed in the partial sequence of the candidate gene (1). The M13 endopeptidases are zinc-containing type II integral membrane glycoproteins with a relatively short cytoplasmic amino-terminal region, a single transmembrane domain, and a long extracytoplasmic domain, which contains the active site of the enzyme (8). In addition to PHEX, this family includes neprilysin (NEP, neutral endopeptidase 24.11), a widely distributed peptidase involved in the degradation of several bioactive peptides (9), the endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2) responsible for the processing of inactive big-endothelins into active endothelins (10), the Kell blood group protein, a protein of the erythrocyte membrane with unknown function (11), and ECEL/DINE (12,13) and SEP/NL1 (14,15), two recently reported peptidases with homology to the neprilysin family.

25

The precise physiological role of PHEX is unknown and the mechanisms whereby loss of PHEX function causes renal phosphate wasting, abnormal regulation of vitamin D metabolism and impaired bone mineralization are not completely understood. Homology of PHEX to members of the M13 family of zinc metallopeptidases suggests a role in regulating the activity of extra-cellular bioactive peptide(s) that act in an autocrine, paracrine or endocrine fashion. In support of this hypothesis, Lajeunesse et al. (16) and

30

Nesbitt et al. (17) have reported the existence of a renal phosphate transport inhibitory factor in the culture medium of osteoblasts isolated from the *Hyp* mouse, an animal model for human XLH (18). The role of PHEX would appear be to inactivate this circulating factor. However, the
5 molecular identity of this factor (phosphatonin) has yet to be established.

PHEX and mineralization. *In situ* hybridization performed on sections of embryos and newborn mice showed the presence of Phex mRNA in osteoblasts and odontoblasts (32). Phex gene expression was detectable
10 on day 15 of embryonic development, which coincides with the beginning of intracellular matrix deposition in bones. Moreover, Northern analysis of total RNA from calvariae and teeth of 3-day-old and adult mice showed that the abundance of the Phex transcript is decreased in adult bones and in non growing teeth but maintained in growing incisors throughout life.
15 This result was confirmed when the presence of the Phex protein in new born and adult bones was investigated by Western blotting using a monoclonal antibody raised against human PHEX. Immunohistochemical studies on a 2 month-old mouse showed exclusive labeling of mature osteoblasts and osteocytes in bones and of odontoblasts in teeth (27).
20 Taken together these results suggest that PEX/Phex plays an important role in the development and maintenance of mineralization in these tissues.

Studies performed with the *Hyp* mouse, an animal model harboring a large
25 deletion in the 3' region of the *Phex* gene (5) and exhibiting the same phenotypic features that characterize patients with XLH (18), also suggest that PHEX is involved, in an unknown way, in the mineralization process. *Hyp* mice exhibit enlarged osteoid area in bones (27, 33) which was shown not to be due to abnormal matrix deposition (34,35) but to impaired
30 mineralization (33).

Osteocalcin. Osteocalcin is the most abundant of the non-collagenous bone proteins and is expressed only in osteoblasts. The mature peptide is 49 amino acids long with 3 Gla residues and one disulfide bond. A vitamin K-dependent gamma-carboxylase is responsible for the transformation of certain Glu into Gla residues. These Gla residues are well known for their affinity toward calcium ions and hydroxyapatite crystals. About 80% of bone mass is made-up of hydroxyapatite, a mineral composed of calcium and phosphate. An increase in osteocalcin mRNA is associated with the mineralization stage and the transformation of osteoblasts into osteocytes (39).

In vitro, osteocalcin inhibits growth of mineral crystals (36, 37) whereas *in vivo*, gene targeting aimed at the disruption of both mouse osteocalcin genes resulted in the generation of osteocalcin-deficient animals (38). These osteocalcin-knockout mutant mice display a phenotype "opposed to osteoporosis" which is also characterized by an increase in the rate of bone formation, increased bone mass, as well as an overall improved functional quality of the bone.

SUMMARY OF THE INVENTION

As suggested above, PHEX activity is required for proper bone formation. Unexpectedly, osteocalcin was found to inhibit the enzymatic activity of PHEX *in vitro*. An object of the present invention is to increase *in vivo* PHEX activity to improve bone characteristics, either by reducing the inhibitory activity of osteocalcin or through the administration of PHEX. The present invention further provides a method for identifying agents capable of increasing (potentiating) PHEX activity by preventing the inhibitory activity of osteocalcin on PHEX. The present invention also provides for improved means of PHEX production and purification as well as for a method of increasing PHEX activity by administering soluble PHEX enzyme by means of injection, gene therapy, where the therapeutic

gene codes for the soluble protein, or alternatively, with cell therapy, where the therapeutic cell produce the soluble protein.

More specifically, the present invention combines the inhibitory capacity of
5 osteocalcin on PHEX activity with the invention disclosed in International
Application Number PCT/CA00/00201 (hereinafter referred to as the "201
method"), to identify agents capable of increasing significantly PHEX
activity. The 201 method provides for the necessary reagents and for the
measurement of PHEX activity. The present invention uses the 201
10 method in the presence of osteocalcin to screen agents capable of
abolishing the inhibitory action of osteocalcin. Consequently, when
osteocalcin inhibitory activity is reduced, PHEX enzymatic activity is
increased (or potentiated). To this end, various reagents were prepared
and tools designed to construct an enzymatic assay. Several
15 improvements to the 201 method are described herein. These
improvements include the use of a new vector to produce secPHEX and
the use of a hydrophobic column as a second purification step to obtain
PHEX having a greater purity. The new vector contains a NL1-PHEX
fusion constructed in such fashion that PHEX sequences are immediately
20 downstream from the NL1 furin cleavage site. NL1 is a peptidase secreted
by cells due to a furin-cleavage site (42) in its extracellular domain (15).
Upon biosynthesis of the fusion protein, PHEX is secreted due to furin
cleavage. The advantage of this system over the one described in the 201
method is that no exogenous amino acid residue is present at the N-
25 terminus of secPHEX.

PHEX enzymatic activity can also be potentiated with the administration of
the soluble form of the enzyme as provided in the 201 method and in the
improvements herein. Moreover, PHEX enzymatic activity can be
30 potentiated with the administration of the soluble form of the inactive
enzyme, as provided herein, where the inactive PHEX (iPHEX) acts as a
decoy to bind osteocalcin and thereby reduces the free concentration of

osteocalcin. Similarly, osteocalcin activity can be reduced with an antibody prepared in such a way to prevent the binding of osteocalcin to PHEX.

In addition, the present invention provides several new substrates
5 necessary for the measurement of the enzymatic activity of PHEX. Another improvement involves the use of a PHEX substrate with a single cleavage site. This simplifies the calculations required to measure the activity of the enzyme. The PHEX substrate can be selected from the following permutations: any peptides selected from the sequence
10 represented by human PTHrP 107-139 where the selected peptide comprises at least 2 but preferably 4 residues on each side of at least one DT or DS amino acid pair. Any selection can be further modified with conservative amino acid substitutions: any hydrophobic amino acid can be replaced by another hydrophobic acid, any acidic amino acid can be
15 replaced by another acidic amino acid, any basic amino acid can be replaced by another basic amino acid, serine can be replaced by threonine and vice versa, asparagine can be replaced by glutamine and vice versa. A person skilled in the art will recognize that amino acid substitutions should be made in such a way that the enzymatic process will not be
20 adversely interfered with, in particular with substitutions that involve proline or glycine. One example of a substrate is AWLDSGV (SEQ ID NO: 1) corresponding to human PTHrP 110-116.

The present invention also relates to compositions for treating bone-
25 related disorders in humans and animals. The present invention particularly provides for the treatment of reduced bone mass, including its most frequent manifestation, osteopenia, osteoporosis, as well as various forms of rickets, including X-linked hypophosphatemic rickets. Also, the present invention particularly provides for the faster regeneration of bone
30 mass after bone fractures, the implantation of orthopedic prostheses and the implantation of dental prostheses.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS OF THE INVENTION

The invention will now be described with reference to the following specific
embodiments and drawings, the purpose of which is to illustrate the
5 invention and not to limit its scope.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure1. Structure and expression of PHEX, secPHEX and
10 secPHEXE581V.

- A. Schematic representation of PHEX protein (PHEX), and amino acid
sequence of the wild-type (TM) and mutated (sec) transmembrane
domains (SEQ ID NO: 2 and SEQ ID NO:3, respectively). The hatched
box indicates the position of the transmembrane domain and the solid
15 box shows the position of zinc-binding amino acids. Amino acid
sequences are presented in the one letter code. In the sec sequence,
bold letters show the position of mutated amino acids whereas dashes
(-) depict deleted residues.
- B. Immunoblot analysis of PHEX, secPHEX and secPHEXE581V
20 expression. Proteins from cellular extracts (c) or culture media (m) of
mock-transfected LLC-PK₁ cells (Mock) or cells transfected with either
PHEX, secPHEX or secPHEXE581V (secPHEXmut) were resolved on
a 7.5% SDS-PAGE gel and visualized with a PHEX-specific antibody
as described in the Materials and Methods. Some samples were
25 treated with PNGase F (F) or endo H (H) before electrophoresis. (-)
refers to untreated samples. The positions of molecular mass markers
are indicated (Mr).

Figure 2. Purification of secPHEX. Proteins present in the culture
30 medium of secPHEX-producing LLC-PK₁ cells (lane 1), or in fractions
pooled after the SP-Sepharose column (lane 2) or after the Butyl
Sepharose 4 column (lane 3) were separated on a 7.5% acrylamide gel

and silver stained. The positions of molecular mass markers are indicated (Mr).

Figure 3. secPHEX activity. HPLC analysis of PTHrP₁₀₇₋₁₃₉ digestion fragments. (A) PTHrP₁₀₇₋₁₃₉ in the absence of secPHEX; (B) PTHrP₁₀₇₋₁₃₉ in presence of secPHEX; (C) PTHrP₁₀₇₋₁₃₉ in the presence of secPHEX and 1 mM EDTA; (D) PTHrP₁₀₇₋₁₃₉ in the presence of secPHEXE581V. Arrows indicate the elution position of PTHrP₁₀₇₋₁₃₉ and asterisks the elution position of Tyr-Gly-Gly used as internal standard.

10

Figure 4. Identification of secPHEX cleavage positions in PTHrP₁₀₇₋₁₃₉. The sequences of PTHrP₁₀₇₋₁₃₉, and fragments identified by mass spectrometry are presented (SEQ ID NO: 4 to SEQ ID NO:8). Cleavage sites are indicated by arrows. The one letter code is used to represent the amino acid residues.

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Figure 5. pH dependency of secPHEX activity. secPHEX and PTHrP₁₀₇₋₁₃₉ were incubated in different pH conditions as described in the text and the extent of hydrolysis of the substrate determined by HPLC. The condition yielding the highest hydrolysis was arbitrarily set at 100%. Squares: assays performed in MES buffer; triangles: assays performed in Tris buffer.

Figure 6. Effects of increasing concentrations of pyrophosphate, osteocalcin, and calcium in the presence of osteocalcin on PTHrP₁₀₇₋₁₃₉ degradation by purified secPHEX. secPHEX activity was measured in the presence of increasing concentrations of pyrophosphate or osteocalcin (A), and CaCl₂ (B)(with a constant osteocalcin concentration of 2x10⁻⁶M). In (A) 100% corresponds to the activity of secPHEX in the absence of inhibitors. In (B), osteocalcin inhibitory potency is measured and 100% corresponds to the inhibition observed in the presence of 2x10⁻⁶M osteocalcin and the absence of CaCl₂.

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Figure 7. Comparison of the effects of increasing concentrations of human-produced and *E. coli*-produced osteocalcin on PTHrP₁₀₇₋₁₃₉ degradation by purified secPHEX. (A) secPHEX activity was measured in the presence of increasing concentrations of human-produced (open symbols) or *E. coli*-produced (solid symbols) osteocalcin. 100% corresponds to the activity of secPHEX in the absence of osteocalcin. (B) osteocalcin inhibitory potency is measured and 100% corresponds to the inhibition observed in the presence of 2×10^{-6} M human-produced (open symbols) or *E. coli*-produced (solid symbols) osteocalcin and the absence of CaCl_2 .

In order to provide a clear and consistent understanding of terms used in the present description, a number of definitions are provided hereinbelow.

- Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).
- The nucleic acid (e.g. DNA or RNA) for practicing the present invention may be obtained according to well known methods.

The term "DNA" molecule or sequence (as well as sometimes the term "oligonucleotide") refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), often in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as the term is

defined herein. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α -nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^3H , ^{14}C , ^{32}P , and ^{35}S . Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will

become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, *Am. Biotechnol. Lab.* 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Q β replicase system and NASBA (Kwoh et al., 1989, *Proc. Natl. Acad. Sci. USA* 86, 1173-1177; Lizardi et al., 1988, *BioTechnology* 6:1197-1202; Malek et al., 1994, *Methods Mol. Biol.*, 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analyzed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel

electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

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Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance
10 with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and *ibid.*, 1992, Nucleic Acids Res. 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a
15 nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence
20 of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

A "heterologous" (e.g. a heterologous gene) region of a DNA molecule is a subsegment of DNA within a larger segment that is not found in
25 association therewith in nature. The term "heterologous" can be similarly used to define two polypeptidic segments not joined together in nature. Non-limiting examples of heterologous genes include reporter genes such as luciferase, chloramphenicol acetyl transferase, β -galactosidase, and the like which can be juxtaposed or joined to heterologous control regions or
30 to heterologous polypeptides.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA

vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

5 The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

10 The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

15

Operably linked (or alternatively, "in frame") sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce
20 an RNA transcript of the reporter sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

25 Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

30 Prokaryotic expressions are useful for the preparation of large quantities of the protein encoded by the DNA sequence of interest. This protein can be purified according to standard protocols that take advantage of the intrinsic

properties thereof, such as size and charge (e.g. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal antibodies. The purified protein can be used for
5 therapeutic applications.

The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the
10 Luciferase reporter molecule. "Promoter" refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to
15 include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic
20 promoters will often, but not always, contain "TATA" boxes and "CCAT" boxes. Prokaryotic promoters contain -10 and -35 consensus sequences, which serve to initiate transcription and the transcript products contain Shine-Dalgarno sequences, which serve as ribosome binding sequences during translation initiation.

25

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether a nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original
30 sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one

or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid generally has chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophilicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (e.g. solubility, absorption, half life, decrease of toxicity and the like). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide or nucleic acid sequence are well known in the art.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted,

substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

5 As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in most other cellular components.

10

As used herein, the terms "molecule", "compound", "agent" or "ligand" are used interchangeably and broadly to refer to natural, synthetic or semi-synthetic molecules or compounds. The term "molecule" therefore denotes for example chemicals, macromolecules, cell or tissue extracts
15 (from plants or animals) and the like. Non limiting examples of molecules include nucleic acid molecules, peptides, antibodies, carbohydrates and pharmaceutical agents. The agents can be selected and screened by a variety of means including random screening, rational selection and by rational design using for example protein or ligand modeling methods such
20 as computer modeling. The terms "rationally selected" or "rationally designed" are meant to define compounds which have been chosen based on the configuration of interacting domains of the present invention. As will be understood by the person of ordinary skill, macromolecules having non-naturally occurring modifications are also within the scope of the term
25 "molecule". For example, peptidomimetics, well known in the pharmaceutical industry and generally referred to as peptide analogs can be generated by modeling as mentioned above. Similarly, in a preferred embodiment, the polypeptides of the present invention are modified to enhance their stability and functionality. It should be understood that in
30 most cases this modification should not alter the biological activity of the interaction domain. The molecules identified in accordance with the teachings of the present invention have a therapeutic value in diseases or

conditions in which the physiology or homeostasis of the cell and/or tissue is compromised by a reduction in active PHEX. Alternatively, the molecules identified in accordance with the teachings of the present invention find utility in the development of increased PHEX activity.

5

As used herein, potentiators of PHEX results from the interaction of molecules where the PHEX activity is enhanced. In one embodiment, potentiators can be detected by contacting the indicator assay with a compound or mixture or library of molecules for a fixed period of time is
10 then determined. An indicator assay in accordance with the present invention can be used to identify potentiators. For example, the test molecule or molecules are incubated with the host cell in conjunction with one or more molecules held at a fixed concentration. An indication and relative strength of the potentiating properties of the molecule(s) can be
15 provided by comparing the level of peptide hydrolysis in the indicator assay in the presence of the agonist, in the absence of test molecules v. in the presence thereof. Of course, the potentiating effect of a molecule can also be determined in the absence of agonist, simply by comparing the level of hydrolysis product in the presence and absence of the test
20 molecule(s).

It shall be understood that the "*in vivo*" experimental model can also be used to carry out an "*in vitro*" assay.

25 As exemplified herein below, the interaction domains of the present invention can be modified, for example by *in vitro* mutagenesis, to dissect the structure-function relationship thereof and permit a better design and identification of modulating compounds. However, some derivative or analogs having lost their biological function of interacting with their
30 respective interaction partner may still find utility, for example for raising antibodies. Such analogs or derivatives could be used for example to raise antibodies to the interaction domains of the present invention. These

antibodies could be used for detection or purification purposes. In addition, these antibodies could also act as competitive or non-competitive inhibitor and be found to be modulators of PHEX or osteocalcin interaction.

5 A host cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting
10 DNA may be maintained on a episomal element such as a plasmid. With respect to eukaryotic cells, a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or
15 clones comprised of a population of daughter cells containing the transfecting DNA. Transfection methods are well known in the art (Sambrook et al., 1989, *supra*; Ausubel et al., 1994 *supra*). The use of a mammalian cell as indicator can provide the advantage of furnishing an intermediate factor, which permits for example the interaction of two
20 polypeptides which are tested, that might not be present in lower eukaryotes or prokaryotes. Of course, such an advantage might be rendered moot if both polypeptide tested directly interact. It will be understood that extracts from mammalian cells for example could be used in certain embodiments, to compensate for the lack of certain factors.

25

In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular
30 Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody- A Laboratory Manual, CSH Laboratories). The present invention also provides polyclonal, monoclonal

antibodies, or humanized versions thereof, chimeric antibodies and the like which inhibit or neutralize their respective interaction domains and/or are specific thereto.

- 5 From the specification and appended claims, the term therapeutic agent should be taken in a broad sense so as to also include a combination of at least two such therapeutic agents. Further, the DNA segments or proteins according to the present invention can be introduced into individuals in a number of ways. For example, erythropoietic cells can be isolated from
10 the afflicted individual, transformed with a DNA construct according to the invention and reintroduced to the afflicted individual in a number of ways, including intravenous injection. Alternatively, the DNA construct can be administered directly to the afflicted individual, for example, by injection in the bone marrow. The DNA construct can also be delivered through a
15 vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different routes.

For administration to humans, the prescribing medical professional will
20 ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen therapeutic regimen (e.g. DNA construct, protein, cells), the response and condition of the patient as well as the severity of the disease.

- 25 Composition within the scope of the present invention should contain the active agent (e.g. fusion protein, nucleic acid, and molecule) in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Typically, the nucleic acids in accordance with the present invention can be administered to mammals (e.g. humans) in doses ranging
30 from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the present invention and are well known in

the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will be adapted by the clinician in accordance with
5 conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.001 to 50 mg/kg/day will be administered to the mammal.

MATERIALS AND METHODS

10 The production of the reagents used in the 201 method is summarised herein to enable a person skilled in the art to recognise useful improvements in this method.

DNA manipulations

15 All DNA manipulations were performed according to standard protocols (19,20) (Ausubel et al., 1988; Sambrook et al., 1989)(Ausubel et al., 1988; Sambrook et al., 1989). Site-directed mutagenesis was performed using a PCR-based strategy as described previously (21).

20 Construction of expression vectors

Human PHEX cDNA was cloned previously (5). For expression of PHEX in cultured mammalian cells, a restriction fragment (SpeI-EcoRV), which contained the entire PHEX coding sequence, was digested, blunted, and
25 subcloned into the mammalian expression vector pCDNA3/RSV (22). This vector also contains the bacterial *neo* gene that confers resistance to the antibiotic neomycin (G418) to cells that express it. The resulting vector, called pCDNA3/RSV-PHEX, encoded a membrane bound form of PHEX.

30 To obtain a soluble form of human PHEX, the signal peptide/membrane anchor domain (SA domain) of the protein was transformed into a

cleavage-competent signal sequence following a strategy resembling that previously described to generate a soluble form of NEP (23). However, in the case of PHEX more genetic manipulations were needed. In addition to the strategy implemented for NEP, four codons had to be deleted in the SA domain of PHEX in addition to introducing hydrophilic amino acid residues. These modifications were achieved by introducing in vector pCDNA3/RSV/PHEX site-directed mutations (8 codons) and deletions (4 codons) by Polymerase Chain Reaction (PCR), using appropriate oligonucleotide primers, as described previously (21) (Fig. 1A).

Vector pCDNA3/RSV/secPHEXE581V which encodes an inactive secPHEX protein bearing the mutation Glu 581 to Val was obtained by site-directed mutagenesis using the same PCR-based strategy referred to above and appropriate oligonucleotide primers.

Vector pCDNA3/RSV/NL1-PHEX which encodes secPHEX fused with NL1, a furin cleavable signal peptide, was obtained by cloning in vector pCDNA3/RSV NL1 nucleotide sequence corresponding to amino acid residues 1 to 63 (15) preceding and in frame with PHEX nucleotide sequence corresponding to amino acid residues 46 to 750 (5).

To produce human osteocalcin in *E. coli*, total RNA was isolated from human osteoblast-like SaOS2 cells and osteocalcin sequences generated by RT-PCR using appropriate oligonucleotide primers. Osteocalcin sequence was verified by sequencing and introduced in pGEX vector (Amersham Pharmacia Biotech) to yield vector pGEX-OST.

Protein production in *E. coli*

For production of osteocalcin in *E. coli*, pGEX-OST vector was introduced in bacterial strain AP401, and induction and purification of the protein

performed as recommended by the supplier of the pGEX vector (Amersham Pharmacia Biotech).

Transfections and cell culture

5

Human PHEX, secPHEX and secPHEXE581V were expressed in LLC-PK₁ cells (Porcine Kidney cells; ATCC No. CRL-1392). To induce the stable expression of these recombinant proteins, appropriate vectors were
10 transfected in LLC-PK₁ cells by the CaPO₄ precipitation method (24). Transfected cells were selected by adding 400 µg/ml G418 (Life Technologies, Burlington, ON, Canada) to the medium and cultured as described previously (25).

15 Immunoblot analysis of PHEX-related proteins in cells and culture media

For immunoblot analysis, confluent cell cultures were incubated for 16 h in DMEM medium (Life Technologies, Burlington, ON, Canada) containing
20 10mM sodium butyrate to enhance expression of the cDNAs which are under the control of the RSV promoter. Cellular proteins were solubilized as previously described (26). Secreted proteins recovered in culture media were concentrated approximately 10 fold on Centriprep-50 columns (Amicon). Proteins (1/50th of the cellular proteins or of the proteins present
25 in the culture medium from one petri dish) were resolved on 7.5% polyacrylamide/SDS gels and detected by immunoblotting using a monoclonal antibody specific to human PHEX, as described previously (27).

30 To determine the glycosylation state of the proteins, samples were incubated prior to electrophoresis with endoglycosidase H (endoH) or peptide:N-glycosidase F (PNGaseF) as suggested by the distributor (New England Biolabs inc., Mississauga, ON, Canada).

Production of secPHEX or secPHEXE581V

To produce large amounts of secPHEX or secPHEXE581V, confluent cells were incubated for 4 days in 199 medium (Life Technologies, Burlington, ON, Canada) supplemented with 2.5 µg/ml insulin, 17.5 µg/ml transferrin, 2 µg/ml ethanolamine, 100 µg/ml soybean trypsin inhibitor and 10 µg/ml aprotinin. Sodium butyrate was present at a concentration of 10 mM. After 4 days, the media were recovered, centrifuged and concentrated on Centriprep-50 columns. Typically, 600 ml of crude spent medium from transfected LLC-PK₁ cells were concentrated to 30 ml before loading onto an ion-exchange column for purification.

Purification of secPHEX or secPHEXE581V

The concentrated medium was loaded, at a flow rate of 2 ml/min, on a 8 ml SP-Sepharose cation-exchange column (Amersham Pharmacia Biotech inc. Baie d'Urfée, QC, Canada) previously equilibrated with 50 mM sodium phosphate pH 6.6 containing 50 mM NaCl. The column was washed at the same flow rate with 10 column volumes of the same buffer and proteins eluted with a 50mM to 1M NaCl gradient. Fractions were analyzed by SDS-PAGE and immunoblotted as described above, and fractions containing secPHEX or secPHEXE581V were visualized by silver staining.

Alternatively, and in preference to the 201 method, the following modifications in the purification method are recommended. Fractions containing secPHEX or secPHEXE581V are pooled and concentrated to approximately 1.5 mg/ml using Centriprep-50 columns. The protein solution is then diluted to 0.1 mg/ml with buffer A (50 mM phosphate pH 7.0 and 1 M ammonium sulfate), centrifuged at 9000g for 15 min and loaded onto a 1 ml Butyl Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech inc. Baie d'Urfée, QC, Canada) at a flow rate of

1ml/min. The column is washed, at the same flow rate, with buffer A to a stable baseline and the proteins are eluted with a 40 ml gradient from 100% buffer A/0% buffer B (50 mM phosphate pH 7.0) to 0% buffer A/100% buffer B. Fractions are analyzed as described above and those
5 containing secPHEX or secPHEXE581V are visualized by silver staining. Fractions containing pure secPHEX or secPHEXE581V are pooled, concentrated and dialyzed against 50 mM phosphate pH 6.5, 150 mM NaCl using Centriprep-50 columns. Protein concentrations are determined using the Bradford method (DC protein assay kit; Biorad, Mississauga,
10 ON, Canada).

Enzymatic assay

One μg of purified secPHEX or secPHEXE581V was incubated with 5 μg
15 of peptide substrate for 30 min at 37 °C in a volume of 200 μl of 50 mM MES (2-(N-morpholino) ethanesulfonic acid) pH6.5, 150 mM NaCl. Peptide PTHrP₁₀₇₋₁₃₉ (human origin and obtained from Bachem, Philadelphia, PA, U.S.A. or Peninsula Laboratories, Belmont, CA, U.S.A.), used as a substrate, was prepared as 1 $\mu\text{g}/\mu\text{l}$ solutions also containing 1 $\mu\text{g}/\mu\text{l}$ of the
20 tripeptide Tyr-Gly-Gly. This latter peptide, which is not a PHEX substrate, was used as an internal standard. Peptide substrate AWLDSGV (Dr Gilles Lajoie, Waterloo Peptide Synthesis C2-360 University of Waterloo, Waterloo, ON Canada) (SEQ ID NO: 1) was digested with secPHEX in the same manner as described above for PTHrP₁₀₇₋₁₃₉. To determine
25 osteocalcin inhibitory potency on secPHEX activity, osteocalcin (Peptide Research Institute, Japan or *E. coli*-produced) was added to the reaction mixture to final concentrations varying from 10^{-8} to 5×10^{-4} M. To determine the effect of agents on the inhibitory activity of osteocalcin, the agents were added to a reaction mixture containing secPHEX, peptide substrate
30 and 5×10^{-6} M osteocalcin. This concentration of osteocalcin yields approximately 75% inhibition of secPHEX activity. Following the incubation period, hydrolysis was stopped by the addition of EDTA to a final

concentration of 5 mM. The potentiating agent was identified when the intact substrate was determined to be present in a lower concentration than in the absence of the potentiating agent (see Figure 6). Results were quantified by comparing, after normalization for the amounts of peptide
5 Tyr-Gly-Gly present in the samples, the areas under the peaks of the substrate.

Identification and quantification of the enzymatic activity of secPHEX on the PTHrP₁₀₇₋₁₃₉ peptide was performed by reverse phase high
10 performance liquid chromatography (RP-HPLC) on a C18 μ Bondapak analytical column (Waters, Mississauga, ON, Canada) with a UV detector set at 214 nm. Peptides were resolved with a linear gradient of 5% B to 85% B in 45 min at the flow rate of 0.4 ml/min [mobile phase A= 0.1% trifluoroacetic acid; mobile phase B= 80% acetonitrile (CH₃CN), 0.1%
15 trifluoroacetic acid].

Identification and quantification of the enzymatic action of PHEX on the AWLDSGV peptide (SEQ ID NO: 1) was performed by reverse phase high performance liquid chromatography (RP-HPLC) on a 5 μ m ZORBAX
20 300SB-C18 analytical column (Agilent Technologies, Mississauga, ON, Canada) maintained at 40°C with a UV detector set at 220 nm. Peptides were resolved with a linear gradient of 30% B to 45% B in 10 min at the flow rate of 1.0 ml/min [mobile phase A= 0.1% trifluoroacetic acid; mobile phase B= 80% acetonitrile (CH₃CN), 0.1% trifluoroacetic acid].

25

The secPHEX digestion products of PTHrP₁₀₇₋₁₃₉ were characterized by mass spectrometry (MALDI Tof) at the McGill University Mass Spectrometry Center.

30

Administration of secPHEX

Purified secPHEX solubilized in 1 mM phosphate pH 7.4, 150 mM NaCl, was administered intravenously daily for 4 or 14 consecutive days through the subclavian vein Hyp males (12 weeks of age). The same number of aged-matched Hyp male mice were treated with vehicle only as controls.

RESULTS

10 Construction and expression of a soluble form of PHEX

To obtain a soluble form of recombinant human PHEX, we first attempted to transform the signal peptide / transmembrane anchor (SA) domain of PHEX into a cleavage-competent signal peptide using the strategy described previously to generate a soluble form of NEP (23). This strategy resulted in the production of a misfolded PHEX protein that remained trapped in the rough endoplasmic reticulum of transfected cells (results not shown). Therefore, an alternate strategy was developed which consisted in the deletion of selected amino acids in the SA domain of PHEX in addition to substitution of hydrophilic amino acid residues for hydrophobic ones (Fig. 1A).

LLC-PK₁ cells were transfected with pCDNA3/RSV expression vectors containing cDNAs for the membrane or soluble forms of PHEX, and permanent cell lines were established as described under Materials and Methods (LLC-PK₁/PHEX and LLC-PK₁/secPHEX cells, respectively, for the membrane-bound and soluble forms). Immunoblotting with a PHEX-specific monoclonal antibody (27) of extracts of LLC-PK₁/PHEX cells revealed a major band of 105 kDa and a minor band of 95 kDa (Fig. 1B, lane 3). No protein was found in the culture medium, as expected for an integral membrane protein (Fig. 1B, lane 4). In contrast, secPHEX appeared in the culture medium as a 100 kDa species (Fig. 1B, lane 6)

with very little enzyme in the cell extract (Fig.1B, lane 5). To characterize the glycosylation state of PHEX and secPHEX, we next submitted the recombinant proteins to deglycosylation by peptide N-glycosidase F (PNGase F) and endoglycosidase H (endo H). PNGase F removes high mannose as well as most complex N-linked oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side chains of the high mannose type found on proteins in the RER but which have not yet transited through the Golgi complex; thus, resistance to endo H can be used as an indication that the protein has traveled through the Golgi complex. PNGase F treatment showed that all PHEX and secPHEX species were N-glycosylated as their electrophoretic mobility increased following digestion (Fig. 1B, compare lanes 7 and 8, 10 and 11, 13 and 14). Treatment of PHEX with endo H resulted in faster migration of the 95 kDa band (Fig. 1B, lane 9), indicating that this PHEX species is probably an underglycosylated RER-associated form. The major 105 kDa band was resistant to endo H digestion (Fig. 1B, lane 9), consistent with a cell-surface expression of the enzyme as was previously shown for a tagged-form of PHEX (7). secPHEX present in the culture medium was also resistant to endo H digestion (Fig. 1B, lane 12), suggesting true secretion of the enzyme. In contrast, secPHEX from cell extract was sensitive to endo H treatment (Fig.1B, lane 15). The results show differences in the glycosylation state of secPHEX from the culture medium and the cellular extract, and suggests that the cell-associated form of secPHEX is an intracellular species that has not traveled through the Golgi complex.

25

NL1 is a peptidase secreted by cells due to a furin-cleavage site in its extracellular domain (15). We have taken advantage of this feature of NL1 to construct a fusion protein formed of NL1 N-terminal region (up to and including the furin-cleavage site (see 42)) and PHEX extracellular domain. Vector pCDNA3/RSV/NL1-PHEX also promoted the secretion of a soluble form of PHEX (Western blot not shown). The advantage of this method over the previous one described above is the possibility to have fewer

30

foreign amino acid residues in N-terminus of secPHEX that could elicit an immunological response.

Purification of secPHEX

5 SecPHEX could be purified to homogeneity using a two-step procedure (Fig. 2). First, the concentrated culture medium (Fig. 2, lane 1) was loaded onto a SP-Sepharose column and the proteins eluted with a 0.05 to 1 M NaCl gradient. secPHEX eluted at 150-200 mM NaCl. We estimated
10 the amount of secPHEX recovered after this first step at about 2 mg per liter of culture. One contaminant protein eluted with secPHEX (Fig. 2, lane 2). This contaminant protein was separated from secPHEX on the Butyl Sepharose 4 column (Fig. 2, lane 3). The final yield of the purification procedure was estimated at approximately 1 mg of purified secPHEX per
15 liter of culture.

Activity of secPHEX

SecPHEX activity was assayed in 50 mM MES pH 6.5 containing 150 mM
20 NaCl (26). Sodium chloride was added to the reaction mixture because we observed that secPHEX precipitated out in solutions containing less than 50 mM salt.

In the absence of secPHEX, no digestion of PTHrP₁₀₇₋₁₃₉ (SEQ ID NO: 4)
25 (elution time 31.5 min) was evident (Fig. 3A). In the presence of secPHEX, however, approximately 75 to 80% degradation of the peptide was observed (Fig. 3B). (The peak eluting at 7 min corresponds to Tyr-Gly-Gly used as internal standard). Digestion of PTHrP₁₀₇₋₁₃₉ by secPHEX resulted in the production of four degradation products eluting at 23.5,
30 24.2, 27.0 and 29.4 min (Fig. 3B). As expected for a zinc metallopeptidase, secPHEX activity was fully inhibited by the addition of 1mM EDTA (Fig. 3C) or 1 mM 1,10-phenanthroline (result not shown) to the reaction mixture.

To confirm that the activity of secPHEX was not due to a contaminant protease co-purifying with it, a mutant of secPHEX, secPHEXE581V, in which the critical catalytic Glu₅₈₁ was replaced by a Val residue, was constructed. A similar mutation introduced in NEP (28) or ECE-1 (29) resulted in total loss of catalytic activity. secPHEXE581V was produced in LLC-PK₁ cells and showed an expression pattern essentially identical to that of secPHEX (Fig 1B, compare lanes 10, 11 and 12 with lanes 16, 17 and 18, respectively). However, in contrast to the wild-type form of the secreted enzyme, purified secPHEXE581V failed to degrade PTHrP₁₀₇₋₁₃₉ under similar conditions (Fig. 3D).

PHEX selectivity

To determine the cleavage site specificity of secPHEX, reverse-phase HPLC peaks corresponding to the degradation products of PTHrP₁₀₇₋₁₃₉ were collected and analyzed by mass spectrometry (MALDI-Tof). Figure 4 depicts the PTHrP₁₀₇₋₁₃₉ fragments identified (SEQ ID NO: 5 to SEQ ID NO: 8). As can be seen from the cleavage sites identified, hydrolysis of the peptide by secPHEX occurred at the amino-terminus of aspartate residues.

The pH optimum for the reaction was determined by progressively increasing the pH of the MES buffer from 5.0 to 7.0 or of a Tris buffer (50 mM Tris.HCl, 150 mM NaCl) from 7.0 to 9.0. Maximum activity was observed at pH 6.5 (Fig. 5). secPHEX activity rapidly decreased at more basic pH values.

Inhibition of secPHEX activity

In previous studies we noticed that NEP activity was sensitive to the presence of Pi in the incubation medium (unpublished results). To
5 determine whether secPHEX had the same sensitivity to Pi, we examined the effect of Pi, from 0.1 to 50 mM, on secPHEX activity and found that 50% inhibition was achieved by 3.5 mM Pi. Since Pi proved to be an effective inhibitor of secPHEX and since pyrophosphate, an alkaline phosphatase substrate, is abundant in bone (41), we also examined its
10 effect on secPHEX activity. Increasing the pyrophosphate concentration from 0.1 to 50 mM demonstrated that 50% inhibition of enzyme activity was achieved at 2.5 mM (Fig. 6A).

secPHEX specificity for aspartate residues (Fig. 4) suggested that the S₁'
15 pocket can accommodate negatively charged side chains of amino acid residues. Although osteocalcin, which contains 3 negatively-charged γ -carboxy glutamic acid residues (Gla), was not degraded by secPHEX (data not shown), it was a potent inhibitor of secPHEX-mediated PTHrP₁₀₇₋₁₃₉ hydrolysis. Fifty percent inhibition of secPHEX activity was
20 achieved at 3.6×10^{-6} M osteocalcin (Fig. 6A).

Restoration of PHEX activity

25 The Gla residues of osteocalcin are known to bind calcium ions (30). We thus examined the effect of Ca²⁺ on the inhibitory action of osteocalcin. By varying the CaCl₂ concentration in the assay from 10⁻⁶ to 10⁻² M, we showed that 5×10^{-3} M Ca²⁺ was necessary to reduce the inhibitory potency of osteocalcin by 50% (Fig. 6B). Ca²⁺ had no effect on secPHEX activity in
30 the absence of osteocalcin (data not shown).

The mechanisms by which osteocalcin and inorganic pyrophosphate function as PHEX inhibitors are still unknown. However, both molecules have negatively charged chemical groups (Gla residues in osteocalcin and phosphate groups in pyrophosphate) that may interact in the same fashion with the enzyme. The observation that Ca^{2+} can prevent PHEX inhibition by osteocalcin suggests the hypothesis that Gla residues are involved in osteocalcin/PHEX interaction. Indeed, it has been shown that vitamin K-dependent γ -carboxylation of osteocalcin glutamic acid residues 17, 21 and 24 is required for Ca^{2+} binding (31). Atkinson et al (40) have shown that Ca^{2+} binding to Gla residues induces a conformational change which may be responsible for the restoration of PHEX activity. To test this hypothesis, human osteocalcin without Gla residues was made in *E. coli* and its inhibitory potency in the absence or presence of Ca^{2+} compared to that of osteocalcin extracted from human bones (with Gla residues). *E. coli*-produced osteocalcin has an inhibitory potency similar to osteocalcin extracted from bones (Fig. 7A) but its activity was not modulated by Ca^{2+} (Fig. 7B). These observations indicate that the Gla residues are not essential for osteocalcin/PHEX interaction but are important for the calcium-induced conformational change of the molecule that results in its inability to inhibit PHEX.

secPHEX injection to Hyp mice

Daily injection of purified secPHEX over 4 days to Hyp male mice significantly (Student t test $p < 0.05$) decreased serum phosphate as a function of dose (see table 1). Hyp mouse and XLH patient have an already low serum phosphate and low bone mass. Our results demonstrate that upon secPHEX administration serum levels are further reduced. This result is consistent with the concept of "hungry bones" where the available phosphate is mobilized from the serum to undermineralized bones.

Table 1: Dose response of secPHEX on serum phosphate

Day 0	Day 4	Dose (mg/kg)
1,26	1,29	0
0,99	1,14	0
1,26	1,22	0
1,36	1,18	1
1,37	1,31	1
1,68	1,23	1
1,25	1,06	1
1,16	0,92	10
1,12	0,82	10
1,39	0,89	10
1,14	0,87	10

In a similar experiment, Hyp male mice were injected with daily doses of 1mg/kg secPHEX for 14 days. In this experiment, the serum alkaline phosphatase levels were significantly reduced toward normal levels (71+/- 11 units) (see Table 2, Student t test $p < 0.05$), indicating a process of normalization of the physiological status of the bones.

Table 2: Alkaline phosphate level at 7 day pre-treatment and after 7 and 14 of daily sec PHEX injection

	Pre-treatment	7 th day of treatment	14 th day of treatment
Control	448	309	247
Control	445	317	276
Control	373	235	232
Control	283	187	198
Control	286	290	257
1mg/kg sec PHEX	292	183	196
1mg/kg sec PHEX	357	235	196
1mg/kg sec PHEX	377	233	208
1mg/kg sec PHEX	348	199	193
1mg/kg sec PHEX	323	246	218

At the same time serum phosphate levels were monitored and found to be at the same level as pretreatment at the 7th and 14th day. This information in combination with the 4 day experiment above, indicates that upon secPHEX administration phosphate levels are first reduced due to the "hungry bone" effect, followed by a slow increase toward normal levels. This time-dependent process of normalization presented here is consistent

with the general knowledge that bone healing occurs over long periods of time.

5 These results confirm that secPHEX can be used to improve conditions in mammals related to low bone mass, including the management of X-linked hypophosphatemia and osteoporosis as well as for patients requiring osteogenesis resulting from orthopedic or dental interventions.

10 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the
15 invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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CLAIMS:

1. A method of preventing or treating a bone-related disorder or conditions that require osteogenesis in mammals comprising administering an effective amount of secPHEX.
5
2. A method as defined in claim 1, wherein said bone-related disorder is one of the following: osteopenia, osteoporosis, rickets, X-linked hypophosphatemic rickets, or conditions including orthopedic intervention and dental intervention.
10
3. A composition for preventing or treating a bone-related disorder or conditions that require osteogenesis comprising an effective amount of secPHEX and a pharmaceutically-acceptable carrier.
15
4. Use of a composition as defined in claim 3 for preventing or treating a bone-related disorder.
- 20 5. An orthopedic implant comprising secPHEX.
6. A dental prosthesis comprising secPHEX.
7. A method of preventing or treating a bone-related disorder in mammals or conditions that require osteogenesis comprising administering an effective amount of a substance capable of binding osteocalcin.
25
8. A method of preventing or treating a bone-related disorder in mammals or conditions that require osteogenesis comprising administering an effective amount of secPHEXE581V.
30

9. A method of preventing or treating a bone-related disorder in mammals or conditions that require osteogenesis comprising administering an effective amount of an antibody specific to osteocalcin.

5

10. A method as defined in claim 7, wherein said bone-related disorder is one of the following: osteopenia, osteoporosis, rickets, X-linked hypophosphatemic rickets, or conditions including orthopedic intervention and dental intervention.

10

11. A composition for preventing or treating a bone-related disorder or condition requiring osteogenesis comprising an effective amount of secPHEXE581V and a pharmaceutically-acceptable carrier.

15

12. Use of a composition as defined in claim 11 for preventing or treating a bone-related disorder.

13. An orthopedic implant comprising secPHEXE581V.

20

14. A dental prosthesis comprising secPHEXE581V.

15. A method of screening agents capable of abolishing the inhibitory action of osteocalcin comprising:

25

selecting said agent;

reacting purified secPHEX with a peptide substrate in the presence of said agent and a concentration of osteocalcin capable of causing a measurable inhibition or, preferably, representing about 75% inhibition;

30

allowing the hydrolysis reaction to occur;

quantifying the reaction products; and

in identical conditions, the concentration of the peptide substrate, or the reaction products, is measured in the presence of said agent and is compared to the concentration of the peptide substrate, or the reaction products, measured in the absence of said agent. An agent is positively identified when the concentration of the peptide substrate is reduced or the reaction products are increased.

10

16. A method as defined in claim 15, wherein quantification of said reaction products is performed by reverse phase high performance liquid chromatography.

15

17. A method as defined in claim 15, wherein the reaction is performed in a solution comprising about 50 mM MES and about 150mM NaCl.

20

18. A method as defined in claim 15, wherein the substrate contains the DS or DT motif and that is cleaved by secPHEX.

25

19. A method of producing secPHEX comprising expressing the in frame fusion of the nucleotide sequence corresponding to amino acid residues 1 to 63 of NL1 with the nucleotide sequence corresponding to amino acid residues 46 to 750 of PHEX.

30

20. A vector comprising the in frame fusion of the nucleotide sequence corresponding to amino acid residues 1 to 63 of NL1 with the nucleotide sequence corresponding to amino acid residues 46 to 750 of PHEX.

21. A method of expressing secPHEX in a cell *in vivo*, comprising:

5

providing an expression vector encoding the in frame fusion of the nucleotide sequence corresponding to amino acid residues 1 to 63 of NL1 with the nucleotide sequence corresponding to amino acid residues 46 to 750 of PHEX;

10

introducing the vector into a cell *in vivo*; and
maintaining the cell *in vivo* under conditions permitting expression of secPHEX in the cell.

22. A method of separating secPHEX from other components using a hydrophobic chromatographic column.

15

23. A method as defined in claim 22, wherein said chromatographic column is a Butyl Sepharose 4 fast flow column.

24. A method of separating secPHEX from other components using an ion-exchange chromatographic column.

20

25. A method as defined in claim 24, wherein said chromatographic column is a SP-Sepharose column.

Figure 1

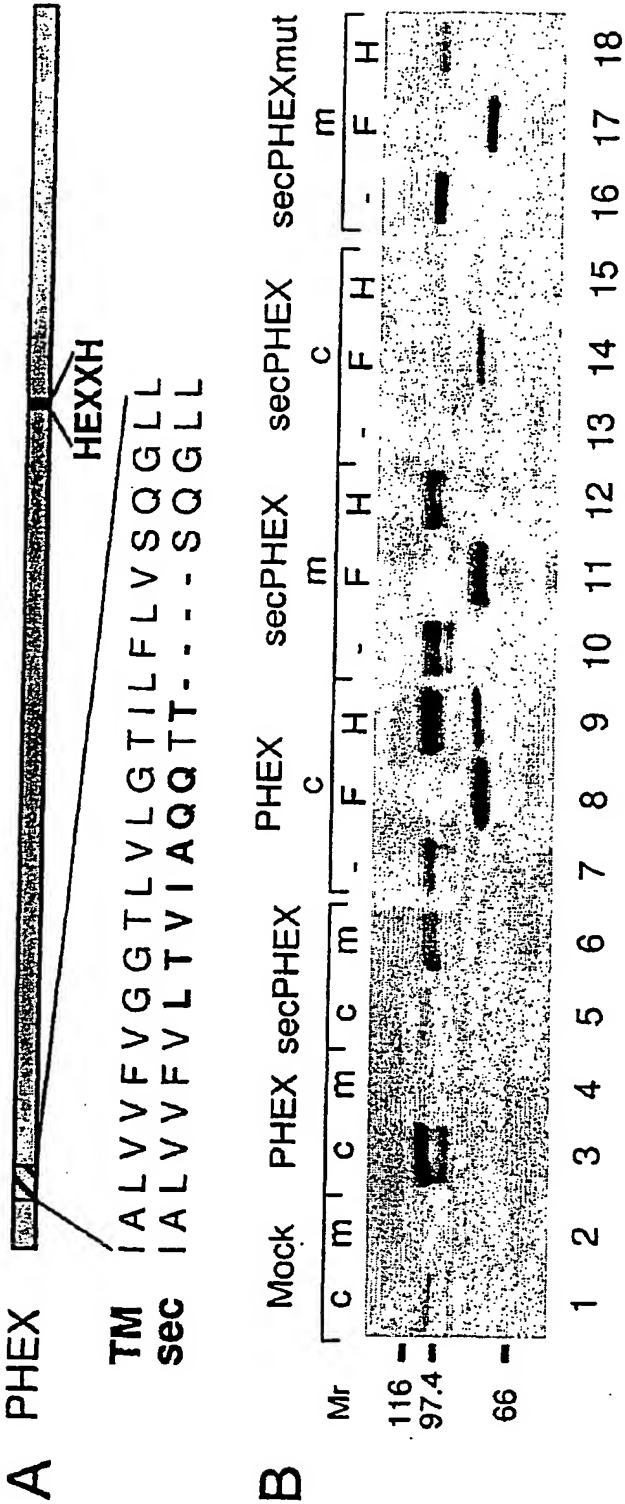
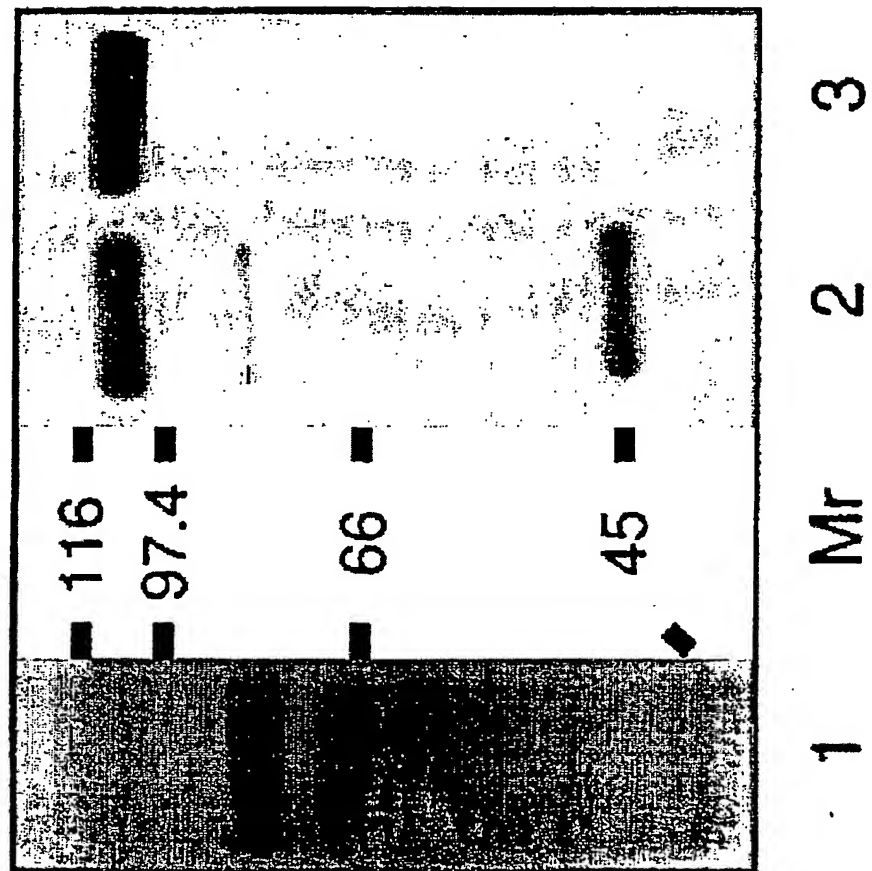


Figure 2



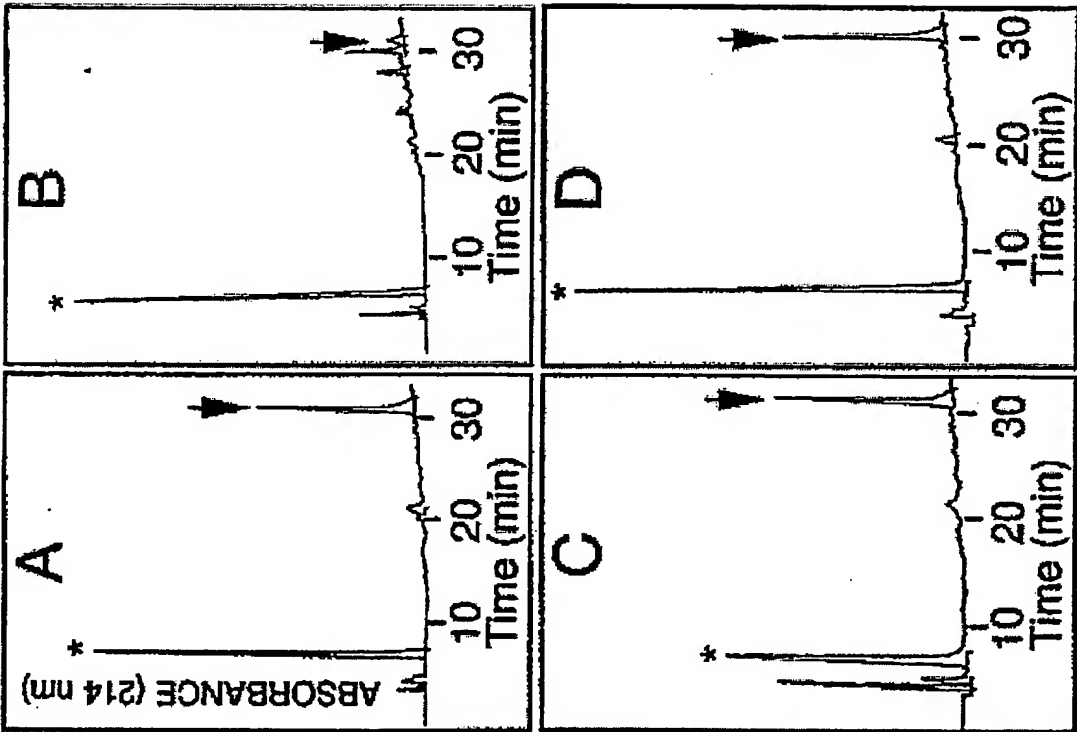
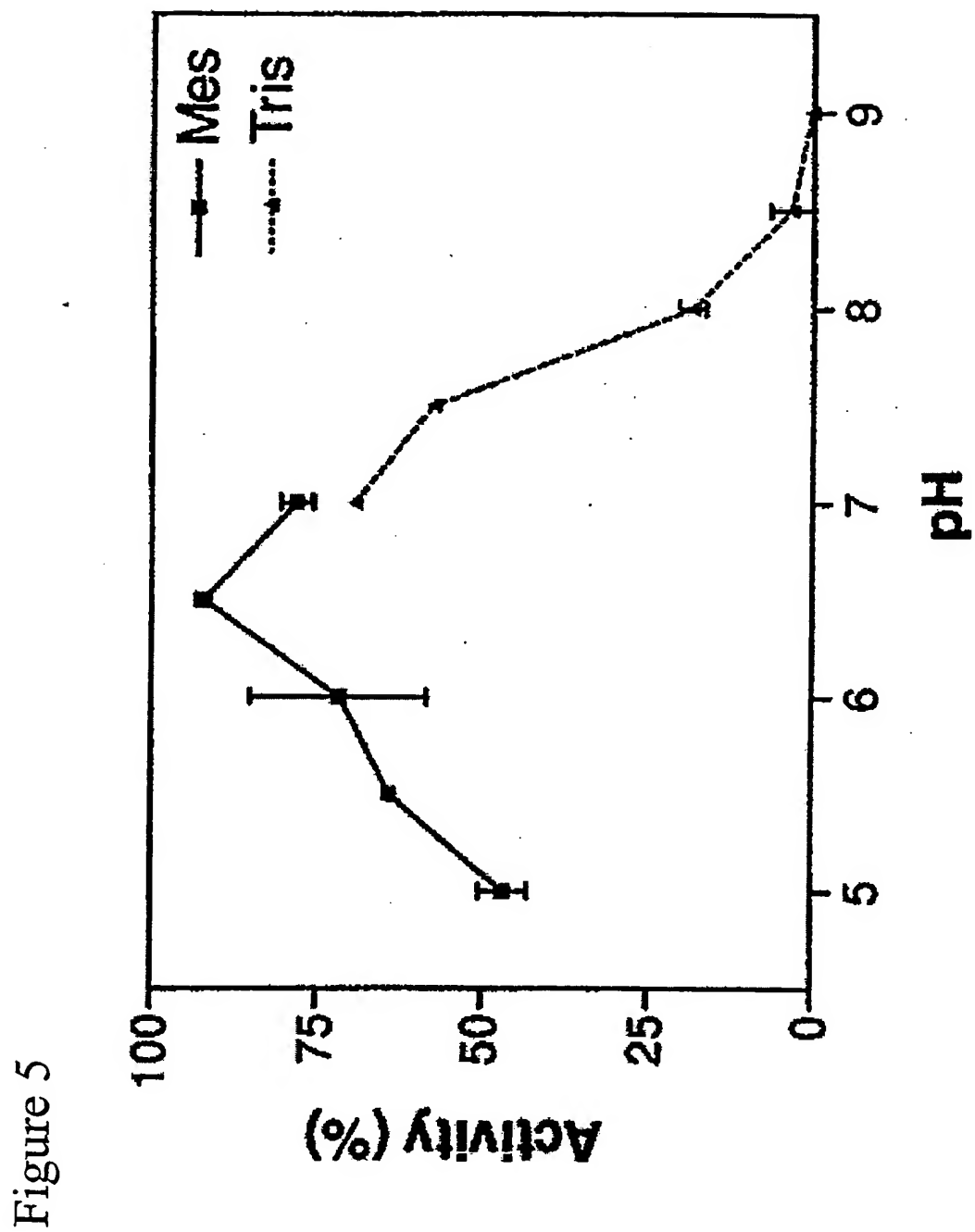


Figure 3

Figure 4

PTHRP(107-139):
 Fragments: (107-112) TRSAWLDSGVTGSGLEGDHLSDTSTTSLELDSR
 (107-127) TRSAWLDSGVTGSGLEGDHLS
 (128-136) DTSTTSLEL
 (128-139) DTSTTSLELDSR



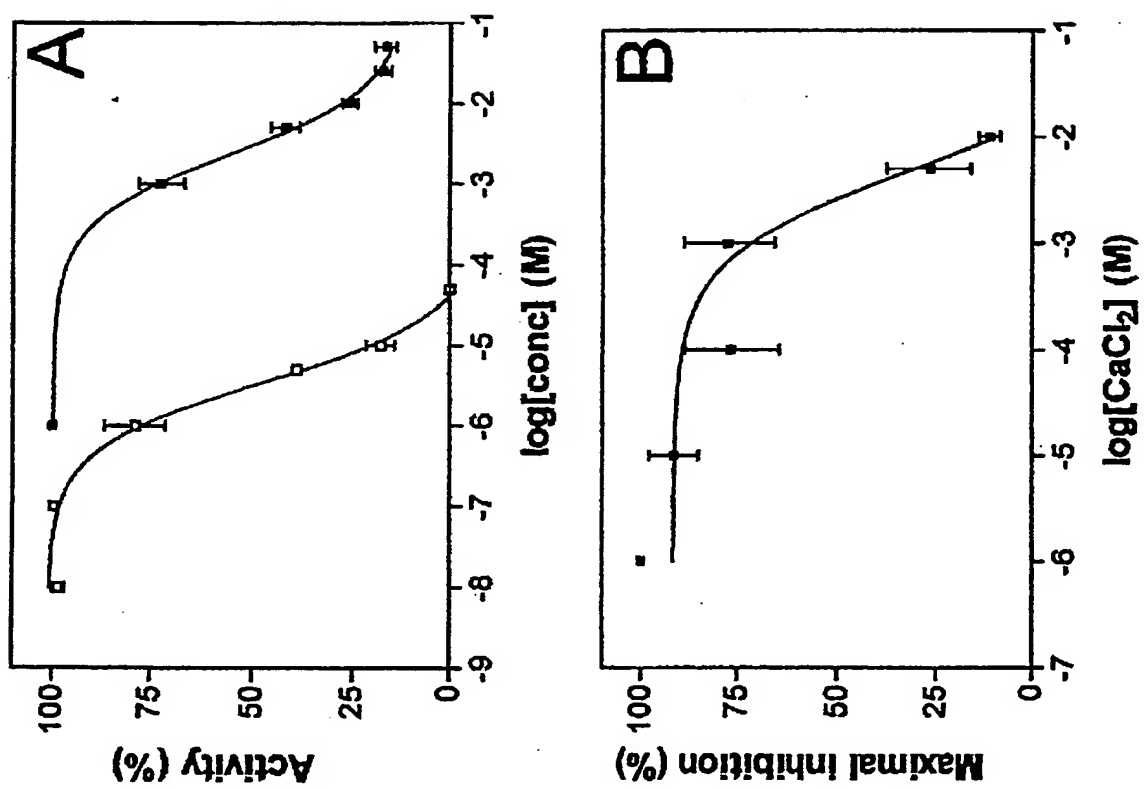
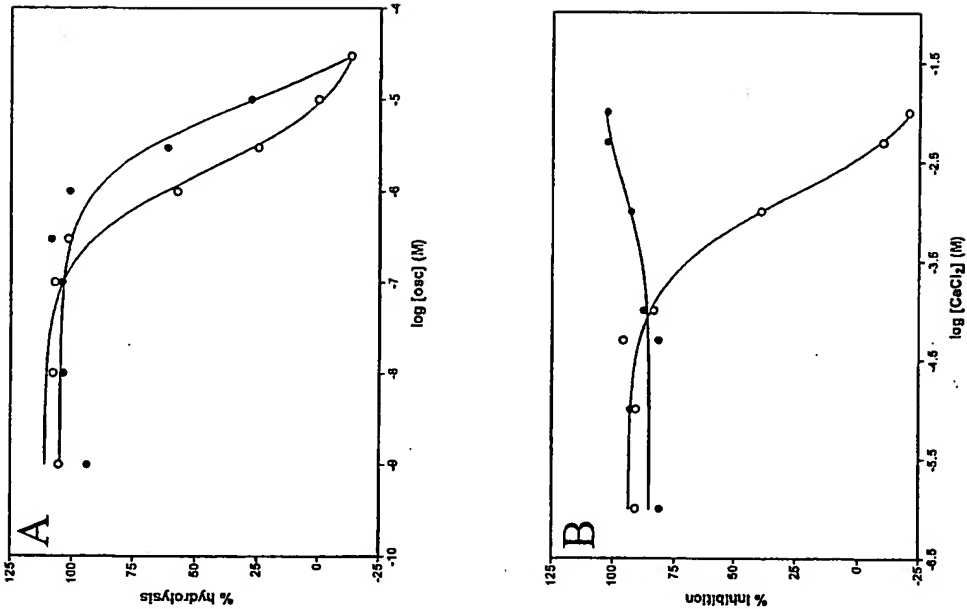


Figure 6

Figure 7



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BOILEAU, Guy

<120> METHOD AND COMPOSITIONS FOR PROMOTING OSTEOGENESIS

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<150> 60/227,012

<151> 2000-08-23

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Leu Phe Leu Val Ser Gln Gly Leu Leu

20

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Ser Gln Gly Leu Leu
20

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<213> Human

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<212> PRT

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1 5 10

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 February 2002 (28.02.2002)

PCT

(10) International Publication Number
WO 02/015918 A3

(51) International Patent Classification⁷: C12N 15/57,
15/62, 9/64, C07K 16/18, G01N 33/68, A61K 38/48,
A61P 19/10

(21) International Application Number: PCT/CA01/01220

(22) International Filing Date: 23 August 2001 (23.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/227,012 23 August 2000 (23.08.2000) US

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

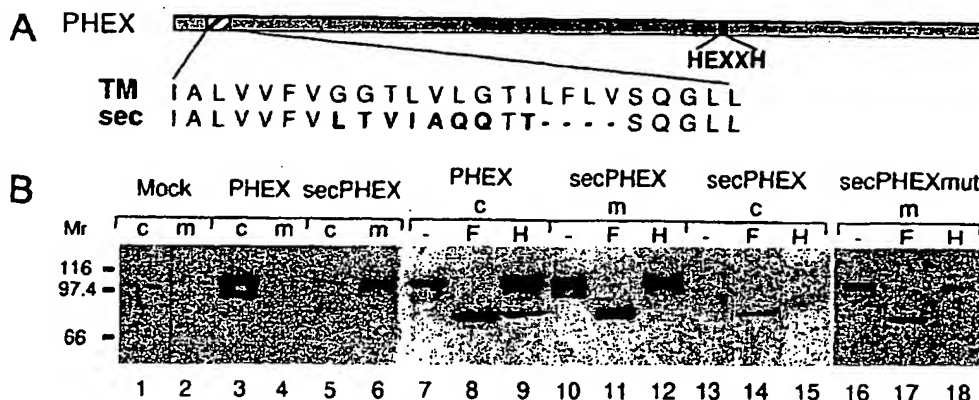
Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

(88) Date of publication of the international search report:
26 September 2002

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD AND COMPOSITIONS FOR PROMOTING OSTEOGENESIS



(57) Abstract: The object of the present invention is to increase PHEX activity to improve the osteogenic process in a mammal where this process has been diminished due to pathology or a condition that require osteogenesis. Osteocalcin is an endogenous inhibitor of the PHEX activity. Therefore, the identification of a substance capable of potentiating PHEX activity by preventing the inhibitory action of endogenous inhibitors will improve osteogenesis. Since PHEX is generally associated with the growth plane of bone or teeth and the absence of osteocalcin is associated with increased bone mass, potentiation of PHEX activity can promote bone growth. Increased PHEX activity can also be obtained by administration of the enzyme itself.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 01/01220

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/57 C12N15/62 C12N9/64 C07K16/18 G01N33/68
 A61K38/48 A61P19/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, SEQUENCE SEARCH, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BOILEAU GUY ET AL: "Characterization of PHEX endopeptidase catalytic activity: Identification of parathyroid-hormone-related peptide107-139 as a substrate and osteocalcin, PPi and phosphate as inhibitors." BIOCHEMICAL JOURNAL, vol. 355, no. 3, 1 May 2001 (2001-05-01), pages 707-713, XP002204778 ISSN: 0264-6021 the whole document	1-4
P,X	WO 00 50580 A (BOILEAU GUY ;UNIV MONTREAL (CA); CRINE PHILIPPE (CA)) 31 August 2000 (2000-08-31) page 21, line 1 - line 18; claims 1-40; figure 1; examples 4,7 ----- -/-	1-4, 24, 25

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

5 July 2002

Date of mailing of the international search report

17/07/2002

Name and mailing address of the ISA

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Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 01/01220

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
P,A	SABBAGH YVES ET AL: "Disease-causing missense mutations in the PHEX gene interfere with membrane targeting of the recombinant protein." HUMAN MOLECULAR GENETICS, vol. 10, no. 15, 2001, pages 1539-1546, XP002204779 ISSN: 0964-6906 the whole document	
A	DE 40 08 546 A (TAKARA SHUZO CO) 20 September 1990 (1990-09-20) the whole document	7,9,10
A	EP 0 504 423 A (TEIJIN LTD) 23 September 1992 (1992-09-23) the whole document	7,9,10
A	EP 0 834 740 A (EISAI CO LTD) 8 April 1998 (1998-04-08) the whole document	7,9,10
A	GHADDAR GALIA ET AL: "Molecular cloning and biochemical characterization of a new mouse testis soluble-zinc-metalloproteinase of the neprilysin family." BIOCHEMICAL JOURNAL, vol. 347, no. 2, 15 April 2000 (2000-04-15), pages 419-429, XP002204780 ISSN: 0264-6021 the whole document	
A	BECK LAURENT ET AL: "Pex/PEX tissue distribution and evidence for a deletion in the 3' region of the Pex gene in X-linked hypophosphatemic mice." JOURNAL OF CLINICAL INVESTIGATION, vol. 99, no. 6, 1997, pages 1200-1209, XP002204781 ISSN: 0021-9738 the whole document	
A	WO 98 10078 A (HENDERSON JANET E ;KARAPLIS ANDREW C (CA); LIPMAN MARK L (CA); SHE) 12 March 1998 (1998-03-12) the whole document	
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 01/01220

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROWE P S N: "THE ROLE OF THE PHEX GENE (PEX) IN FAMILIES WITH X-LINKED HYPOPHOSPHATAEMIC RICKETS" CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION, RAPID SCIENCE, LONDON, GB, vol. 7, no. 4, 1998, pages 367-376, XP001002174 ISSN: 1062-4813 the whole document	
A	ROWE P S N: "THE PEX GENE: ITS ROLE IN X-LINKED RICKETS, OSTEOMALACIA, AND BONE MINERAL METABOLISM" EXPERIMENTAL NEPHROLOGY, KARGER, DE, vol. 5, 1997, pages 355-363, XP000862824 ISSN: 1018-7782 the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 01/01220

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-4, 8-12 and (7,10)-partially are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: (7,10)-partially
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: (7,10)-partially

Present claims 7 and 10 relate to a method of preventing or treating a bone-related disorder in mammals or conditions that require osteogenesis comprising administering an effective amount of a substance capable of binding osteocalcin without giving a true technical characterization. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to antibodies specific to osteocalcin, secPHEX and secPHEXE581V.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 01/01220

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 0050580	A	31-08-2000	AU	2790000 A	14-09-2000
			WO	0050580 A2	31-08-2000
			EP	1159412 A2	05-12-2001
DE 4008546	A	20-09-1990	JP	2242696 A	27-09-1990
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			EP	0834740 A1	08-04-1998
			WO	9738309 A1	16-10-1997
WO 9810078	A	12-03-1998	AU	4107397 A	26-03-1998
			WO	9810078 A2	12-03-1998

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